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Spread of Colorectal Cancer Micrometastases in Regional Lymph Nodes by Reverse Transcriptase-Polymerase Chain Reactions for Carcinoembryonic Antigen and Cytokeratin 20

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Background and Objectives: Lymph node metastasis is known as a significant predictor of prognosis in colorectal cancer patients. Recently, reverse transcriptase polymerase chain reaction (RT-PCR) has been applied to detecting micrometastasis. To assess the risk of recurrence and accurately determine the spread of tumor cells, we examined lymph node micrometastases in a series of colorectal cancer patients.
Methods: We examined 202 lymph nodes obtained from 13 colorectal cancer patients who underwent curative operation and were histologically diagnosed to be node-negative, using RT-PCR to amplify mRNAs for two epithelial markers, carcinoembryonic antigen (CEA) and cytokeratin 20 (CK-20).
Results: All the cases, including early stage patients, had micrometastases. A total of 102 among 202 lymph nodes (50.5%) were positive for either CEA or CK-20, or both (47.0, 40.1, and 36.6% respectively). Positive lymph nodes were spread along the courses of vascular trunks as well as being located in more distant regions.
Conclusions: Even in histologically negative lymph nodes, there is a considerable possibility that micrometastases may exist. Their detection by RT-PCR may improve clinical staging and indications for cancer therapy. We should also take care in the choice of surgical approach.
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KEY WORDS: colorectal cancer; lymph node micrometastasis; reverse transcriptase-polymerase chain reaction; carcinoembryonic antigen; cytokeratin 20

INTRODUCTION

Colorectal cancer is one of the most common malignancies in the world. With progress in diagnostic and therapeutic techniques, its prognosis has been improving [1,2]. It is well established that lymph node metastasis is a significant predictor of a poor prognosis in colorectal cancer [3], but even in cases where no such metastases are histologically apparent, about 20% of patients suffer relapse in less than 5 years [4]. Thus it is important to accurately assess the degree and spread of lymph node metastases as early as possible, to optimize surgical strat-

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TABLE I. Thirteen Node-Negative Cases of Colorectal Cancers: Clinicopathological Findings*

Case	Age/Sex	Location	Tumor size (cm)	Depth of invasion	Histology	Stage
1	71/F	A	3.5 × 2.5	submucosa	Well	I
2	62/F	Rs	5.0 × 4.5	proper muscle	Well	1
3	72/F	T	7.9 × 4.7	subserosa	Well	II
4	42/M	R	5.0 × 4.5	beyond the adventitia	Mod	IIIa
5	63/F	Rs	7.1 × 4.5	subserosa	Mod	II
6	50/F	S	6.0 × 6.0	subserosa	Mod	II
7	85/M	Rs	5.4 × 4.3	subserosa	Well	II
8	70/M	A	5.0 × 3.5	subserosa	Mod	II
9	68/M	S	3.0 × 2.0	subserosa	Well	II
10	70/M	A	5.0 × 3.0	subserosa	Well	II
11	69/M	A	3.0 × 2.5	proper muscle	Well	I
12	69/F	S	3.5 × 3.5	subserosa	Well	II
13	79/F	A	3.5 × 3.0	subserosa	Mod	II

*A, ascending colon; Rs, rectosigmoid colon; T, transverse colon; R, rectum; S, sigmoid colon; Well, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma.

egy and pre-, intra-, or postoperative radioimmunochemotherapy.

Lymph node metastases are usually detected by histological analysis of one or more hematoxylin-eosin sections through each lymph node resected. However, this routine method sometimes fails to detect micrometastases. In the past, immunohistological procedures have been used as an aid to visualization of small foci of cells from gastrointestinal malignancies [5–8], but more recently, more rapid, specific, and sensitive genetic methods including reverse transcriptase polymerase chain reaction (RT-PCR) have been introduced [9,10]. Hayashi et al. [11] have applied mutant allele-specific amplification to detect K-ras and/or p53 altered cells in lymph nodes. However, while useful, this method is restricted to those tumors exhibiting such mutations. In the search for a more general marker, Mori et al. [12] reported that RT-PCR for carcinoembryonic antigen (CEA) mRNA might have advantages for detecting lymph node micrometastases from carcinomas. Another marker of epithelial cells, cytokeratin 20 (CK-20), has also been considered suitable for this purpose [13–15]. However, practical experience with these markers is still rather limited. We therefore examined lymph nodes, diagnosed to be histologically negative for metastases, from a series of colorectal cancer patients, using RT-nested PCR to amplify CEA and CK-20 mRNAs.

PATIENTS AND METHODS

Samples and Cell Lines

Thirty colorectal cancer patients without distant metastasis had curative operation in the Second Department of Surgery, Gifu University, between June 1996 and January 1997. At surgery, we obtained cancer and normal tissues, and regional lymph nodes. The 13 patients who were histologically diagnosed as node-negative were chosen for further study. Six were men and seven were women, and their ages ranged from 42 to 85 (mean 66.9) years of age. Clinical and histological staging [16] was as follows: stage I, 3; stage II, 9; stage IIIa, 1 (Table I).

In each case, the carcinoma and normal tissue more than 10 cm distant were immediately excised and immediately frozen in liquid nitrogen. Storage was at −80°C until extraction of RNA.

A total of 202 lymph nodes could be excised from the 13 patients (average 15.5/case). They were all histologically proven to be free of tumor cells by experienced pathologists. As controls, 12 lymph nodes obtained from five patients with benign disease (cholelithiasis, 2; adhesive ileus, 1; perforated duodenal ulcer, 1; hiatus hernia, 1) and 10 lymph nodes, diagnosed to be histologically positive, from two patients with colorectal cancer were used. At surgery, each lymph node was cut into two with a scalpel, one-half being sent for routine histological examination and the other frozen in liquid nitrogen for subsequent extraction of RNA. To avoid cross contamination, lymph nodes were dissected free from other tissues before opening the intestinal lumen, and a fresh scalpel was used each time.

Colorectal cancer cell lines (SW1083, SW1222, and THRC1) were also investigated as positive controls for the detection sensitivity in these experiments.

RNA Extraction

Frozen samples were ground with a hammer and homogenized. Total cellular RNA was extracted with Isogen (Nippon Gene, Osaka, Japan) by a modified acid guanidinium thiocyanate-phenol-chloroform method [17]. RNA recovery and its purity were determined by Ultrospec 2000 (Pharmacia, Cambridge, England).

Reverse Transcription

cDNA was synthesized from 2 μg total RNA in a 20 μL reaction mixture containing 4 μL of 5 × reaction

buffer (50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, and 3 mmol/L $MgCl_2$), 500 μmol/L dNTP, 100 μmol/L solution of random primers, and 400 units of Moloney Leukemia Virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The mixture was incubated at 42°C for 60 min, heated to 90°C for 2 min, and then chilled on ice.

Sequences of the Primers Used for Nested PCR

The primer sequences for CEA mRNA were: A, 5′-TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3′ for the outer sense; B, 5′-TGTAGCTGTTGCAAATGCTT-TAAGGAAGAA-3′ for the antisense; and C, 5′-GGGC-CACTGTCGGCATCATGATTGG-3′ for the inner sense cases [18]. The primers for the CK-20 mRNA were: D, 5′-CGTCTAACAGTGGAAGCTGATCTC-3′ for the outer sense; E, 5′-TCGGGCGTTCCATGTTAC-TC-3′ for the outer antisense; F, 5′-AAGCATCTGGG-CAACACTGTCA-3′ for the inner sense; and G, 5′-AA-CGGGCCTTGGTCTCCTCTA-3′ for the inner antisense cases.

We checked integrity of the extracted RNA by PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [19] using 5′-ATGGCACCGTCAAGGCTGAG-3′ sense and 5′-CGCCTGCTTCACCACCTTCT-3′ antisense primers.

Nested PCR

We employed two-step PCR for the amplification of CEA cDNA (hemi-nested) and CK-20 cDNA (nested) to enhance the specificity. As reported previously by Gerhard et al. [18], the first PCR was performed using primers A and B followed by the second PCR using primers B and C for the amplification of CEA cDNA. The PCR product was a 132-bp DNA fragment. For the amplification of CK-20 cDNA, the first PCR was performed using primers D and E followed by the second PCR using primers F and G. The product was 313 bp in length.

The nested PCR was performed as follows: for the first PCR, 2 μL aliquots of cDNA solution were mixed with 10.5 μL of PCR reaction mixture containing 1.25 μL 10 × PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, and 1.5 mmol/L $MgCl_2$), 200 μmol/L dNTP, 0.5 μmol/L of each primer, and 0.625 unit of Taq DNA polymerase (Takara Biomedicals, Otsu, Japan). The reaction was continued for 20 cycles (CEA: 95°C, 1 min; 72°C, 2 min; CK-20: 95°C, 1 min; 67°C, 1 min; 72°C, 1 min) with a final step for 10 min in a PCR thermal cycler MP (Takara Biomedicals, Otsu, Japan). One microliter of the first PCR product was then transferred into a second tube, and amplified by 20 cycles (CEA: 95°C, 1 min; 69°C, 1 min; 72°C, 1 min; CK-20: 95°C, 1 min; 67°C, 1 min; 72°C, 1 min) with a final step for 10 min. In preliminary studies, strong CEA band was detected in carcinoma samples with 15 or more cycles, while only a very faint band was evident in a few of the normal control samples after more than 25 cycles. Consequently, we determined 20 cycles to be adequate for the second PCR of CEA. In the CK-20 case, a strong band was also seen in carcinoma samples with 15 or more cycles, while none was apparent for normal lymph nodes after 30 cycles. Therefore 20 cycles were again concluded to be sufficient for the second PCR. We examined each lymph node at least twice.

To confirm the integrity of the cDNA, 25 cycles were carried out for amplification of the GAPDH 626 bp cDNA fragment (94°C, 30 sec; 54°C, 60 sec; 72°C, 80 sec).

The PCR products were subjected to electrophoresis on 1% (GAPDH) and 2% (CEA, CK-20) agarose gels and stained with ethidium bromide. All results were transilluminated and photographed by FAS II (Toyobo, Osaka, Japan).

The amplified products were sequenced using a fmol DNA Cycle Sequencing System (Promega, Madison, WI), and determined to be identical to those expected.

Serial Dilution of Carcinoma Cells, cDNA of CEA and/or CK-20 Predominant Tumors

To determine the sensitivity of the methods, decreasing numbers of SW1083 and SW1222 ranging from 10^5 to 10^0 were added to 10^7 lymphocytes isolated from healthy volunteers. After the extraction of total RNA, RT-nested PCR was performed.

RESULTS

Carcinoma, Control Samples and Cell Lines

All the carcinoma and normal tissue samples, and cell lines demonstrated positive bands for both CEA and CK-20. Positive control lymph nodes with histologically evident metastases also demonstrated both bands. However, all the lymph nodes obtained from the patients with benign disease showed no bands at the same condition (Fig. 1).

No bands were seen in cell lines even if the RT-nested PCR was performed without RT enzyme, which indicated no existence of genomic DNA causing positive bands.

These results demonstrated that the PCR conditions were appropriate.

Serial Dilution

CEA mRNA was detected at a concentration as low as 10^1 tumor cells/10^7 lymphocytes, either CK-20 mRNA was in SW1083 and SW1222 (Fig. 2). Very faint bands were seen at a concentration of 10^1 tumor cells/10^7 lymphocytes. The limits of sensitivity were one tumor cell/10^6 lymphocytes for both CEA and CK-20.

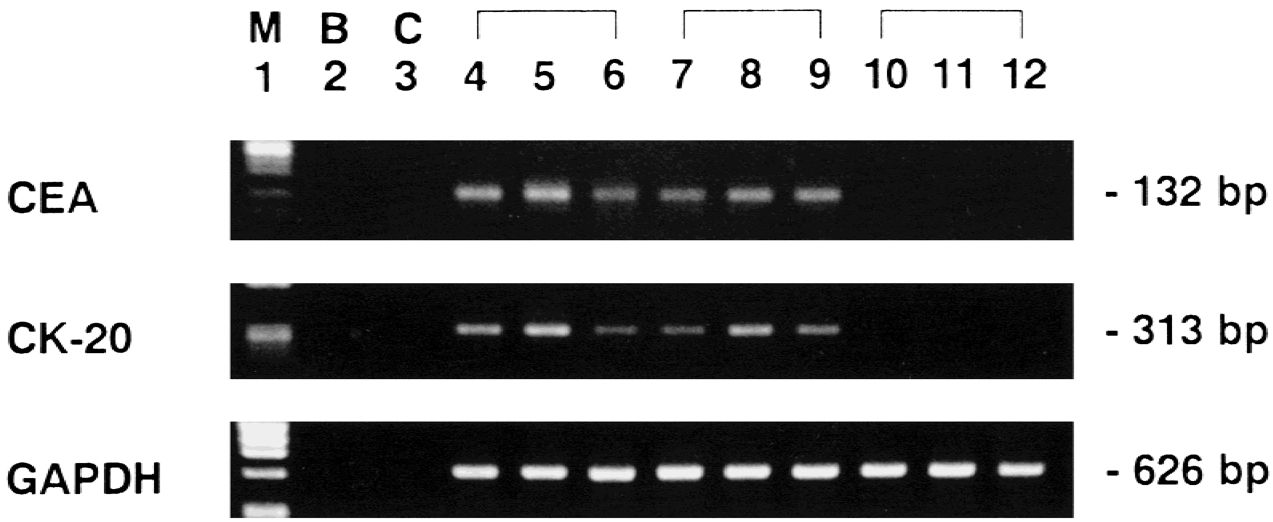

Fig. 1. RT-nested PCR amplification of CEA, CK-20, and GAPDH mRNAs. CEA and CK-20 positive bands are apparent for the three colorectal cancer cell lines (lane 4, SW1083; lane 5, SW1222; lane 6, THRC1) and three histologically positive lymph nodes (lanes 7, 8, 9) but not those obtained from benign disease cases (lanes 10, 11, 12). M, marker (ϕX174/HaeIII digest); B, blank; C, negative control.

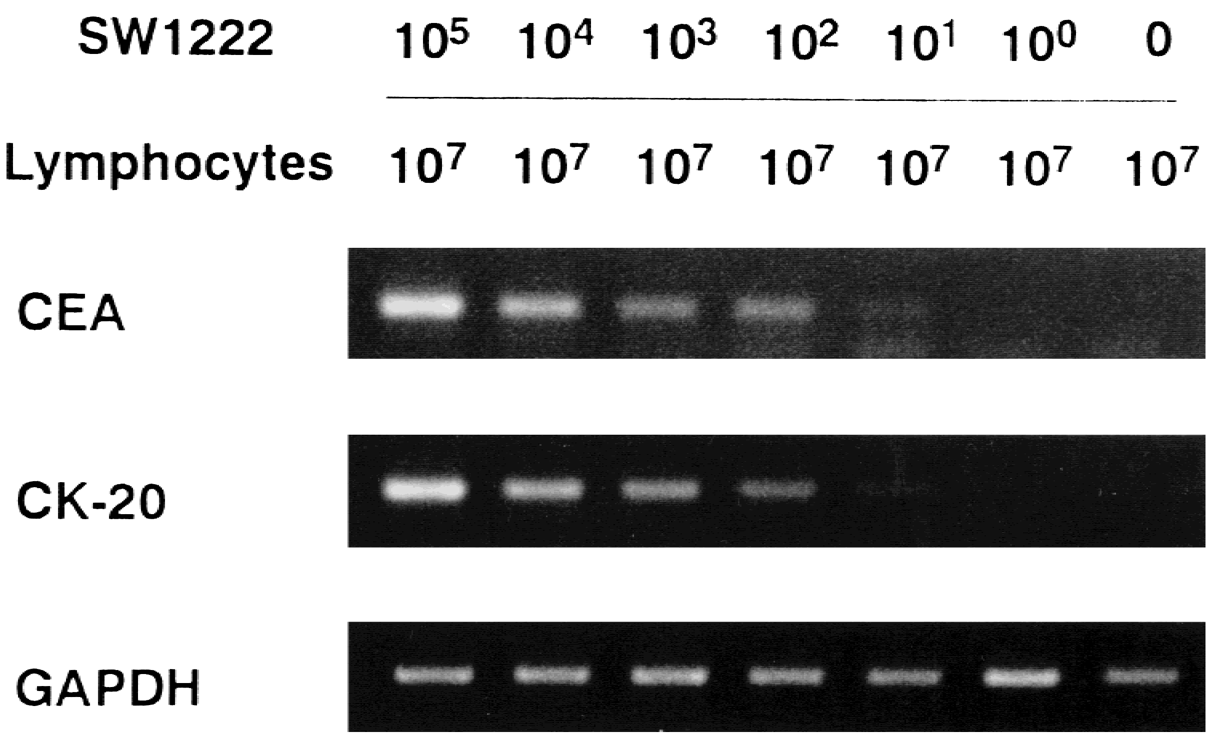

Fig. 2. Serial dilution study using normal lymphocytes to determine the detection sensitivity of the CEA and CK-20 RT-nested PCR. Decreasing numbers of cells of the cell line SW1222 ranging from 10^5 to 10^0 were added to 107 normal lymphocytes obtained from healthy volunteers. Positive bands decreased in accordance with the dilution. Very faint bands were seen at a concentration as low as 10^1 cancer cells per 10^7 lymphocytes in both CEA and CK-20 cases. Under the conditions of this experiment, the sensitivity was one cancer cell per 10^6 lymphocytes.

Lymph Node Samples

Data for detection of CEA and CK-20 mRNAs are summarized in Table II. A total of 102 of 202 lymph nodes examined (50.5%) demonstrated either CEA or CK-20 bands or both. The respective percentages for these cases were 47.0, 40.1, 36.6 (95, 81, and 74 of the 202), with 77.9% of CEA positive lymph nodes (74/95) being CK-20 positive, and 91.4% of CK-20 positive lymph nodes (74/81) being CEA positive. Twelve of 13 patients had more than four positive lymph nodes. Two patients (Cases 7 and 9) exhibited both bands in all ex-

TABLE II. Numbers of Positive Lymph Nodes Detected by RT-PCR for CEA and CK-20*

Case	Examined lymph nodes	Numbers of positive lymph nodes			
		Total	CEA and CK20	CEA only	CK-20 only
1	5	4	3	1	0
2	6	5	5	0	0
3	22	5	3	2	0
4	44	7	7	0	0
5	14	3	1	0	2
6	11	11	9	2	0
7	9	9	9	0	0
8	11	9	7	1	1
9	6	6	6	0	0
10	21	9	4	2	3
11	22	9	7	1	1
12	17	14	7	7	0
13	14	11	6	5	0
Total	202	102	74	21	7

*RT-PCR, reverse transcriptase polymerase chain reaction; CEA, carcinoembryonic antigen; CK-20, cytokeratin 20.

amined lymph nodes. Four of the 13 (Cases 2, 4, 7, and 9) showed no difference in positive number between CEA and CK-20.

In each case, the distribution of CEA and/or CK-20 positive lymph nodes extended not only to the pericolic or perirectal region but also along the course of the adjacent vascular trunks to more distant regions (Figs. 3 and 4). Forty-two of 67 N1 lymph nodes (62.7%), 40 of 65 N2 lymph nodes (61.5%), and 20 of 70 N3 or more distant lymph nodes (28.6%) were respectively positive (Table III).

DISCUSSION

The present study showed that all of 13 colorectal cancer patients with histologically negative lymph nodes actually demonstrated evidence of micrometastases in a large proportion of the nodes sampled. This might explain the findings that about 20% of patients diagnosed to be histologically node-negative demonstrated tumor recurrence in less than 5 years [4]. Thus expansion of residual cancer cells could have been responsible.

Recently various genetic methods have been introduced to detect occult cancer cells. RT-PCR is a rapid, sensitive, and specific technique, which has been successfully applied to detection of micrometastases from a variety of malignant tumors; for example prostatic cancers [20], melanomas [21,22], breast cancers [23,24], hepatocellular carcinomas [25] and gastrointestinal carcinomas [10,12]. In the absence of any specific marker for the latter, we choose CEA and CK-20 as suitable targets for RT-PCR in the present cases because they are known to be commonly highly expressed in colorectal cancers [12,26].

CEA is in fact widely accepted as a useful tumor marker for surveillance of gastrointestinal cancer patients, especially those with colorectal neoplasm [26,27]. RT-nested PCR using CEA has proven to have advantages for detecting micrometastases in bone marrow [18,28] and lymph nodes [12]. Cytokeratins are major cellular proteins with many subgroups [29,30], and some species, such as CK-8,18,19, have also found application for RT-PCR studies of gastric [10], breast [24], lung [31] cancers. However, their specificity is suspect because they often show false positive bands probably derived from pseudogenes. On the other hand, CK-20 appears to be relatively specific for gastrointestinal adenocarcinomas [13–15]. Since RT-PCR (one or two step) has successfully been applied for detection of micrometastases in bone marrow [32,33] and lymph nodes [34], it was adopted for the present study. We also took advantage of the fact that CEA and CK-20 are constitutively expressed in normal colon mucosa and most colorectal cancers, but not in lymphatic tissue [12,35], so that the ectopic presence of their mRNAs would be convincing evidence of occult cancer cells provided that cross contamination was avoided. Therefore suitable precautions were taken when the specimens were excised.

With regard to the sensitivity, we could detect more positive lymph nodes with each marker if reaction cycles were increased. However, the PCR conditions for CEA were restricted by the few false positive bands for normal lymph nodes observed with 25 cycles of the second amplification. No such problem was observed with CK-20 in line with the report that migrating macrophages containing phagocytosed cancer cells are CEA but not CK positive on immunohistochemical staining [5]. Therefore, CK-20 might be the more useful and specific of the two markers but further examination is needed to confirm this. The sensitivity of one cell in 10^6 achieved might explain the surprisingly high frequency of positive lymph nodes. According to our results, all the cases should have been upstaged to Stage III or more, but it may be that not all genetically positive lymph nodes give rise to recurrence. At present we still have no good therapeutic protocols for such patients, but it is clearly important that we should carefully follow them up for the spread of genetic micrometastases.

Surgery is still the most common and effective therapeutic mode for colorectal cancers and the significance of lymphadenectomy is well recognized [1,36]. Recently, various modified operations including autonomic nerve preservation [37,38] and laparoscopic-assisted approaches [39,40] have been performed in consideration of quality of life. Despite the strict indications adopted in deciding candidates for such operations, some cases demonstrated recurrence [41] and this could have been derived from lymph node metastases. We should select appropriate therapeutic modes considering the relationship between the extent of the lymphadenectomy and lymph node micrometastases.

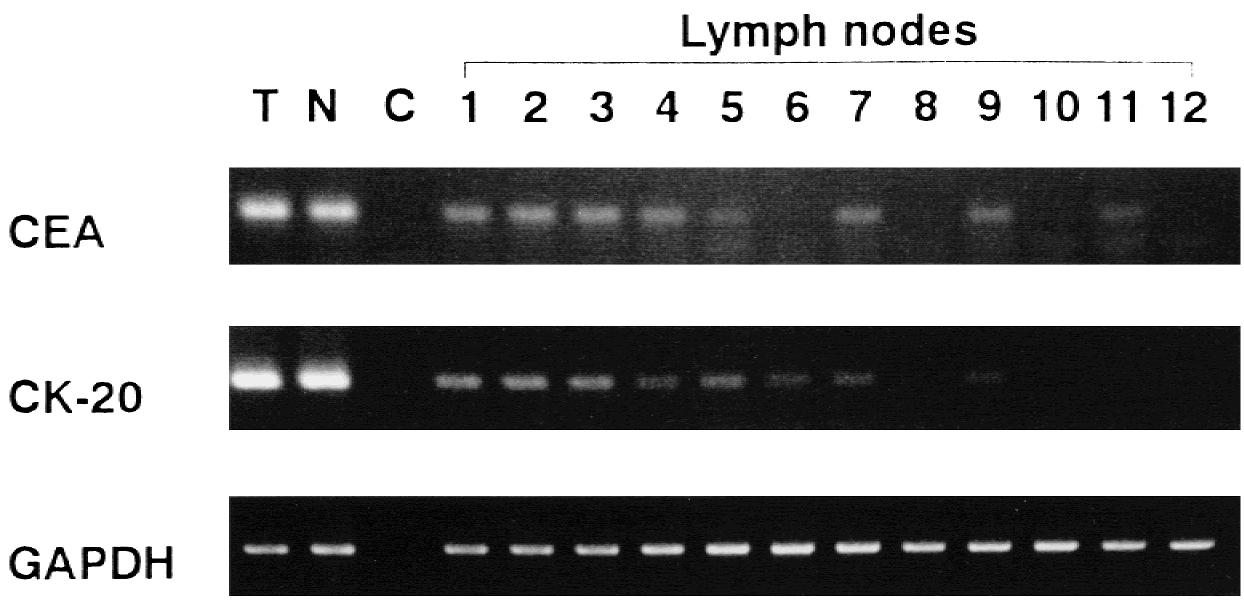

Fig. 3. Findings for 12 of the 22 excised lymph nodes in Case 11 are demonstrated. Lymph node samples numbered 1 to 5 were pericolic lymph nodes, numbers 6 to 8 were those along the course of the right colic artery, numbers 9 to 11 were those at the root of the right colic artery, and number 12 was on the ileocolic artery. Seven lymph nodes (1, 2, 3, 4, 5, 7, 9) were both CEA and CK-20 positive. Number 6 was CK-20 positive only, number 11 was CEA positive only. T, tumor tissue; N, normal tissue; C, negative control.

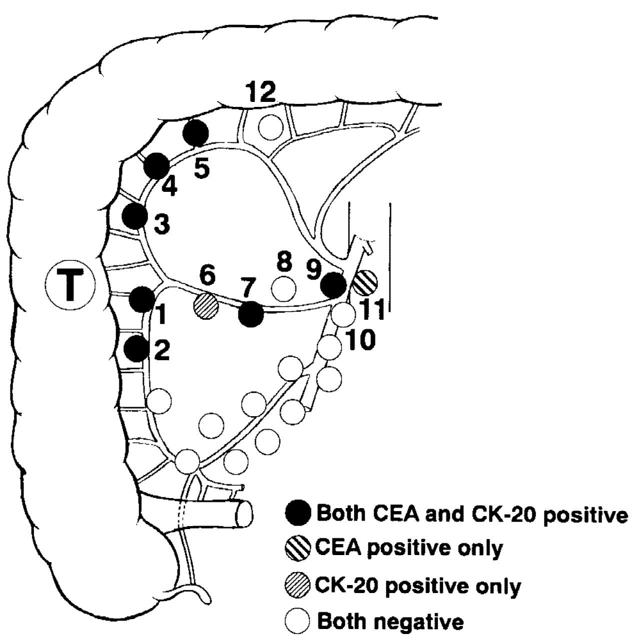

Fig. 4. Lymph node mapping for Case 11. The numbers on the map correspond with the lymph node numbers in Figure 3. Micrometastases are extensive in nodes along the major blood vessels leading from the tumor site despite not being histopathologically detectable.

TABLE III. Distribution of Examined Lymph Nodes

Examined lymph nodes	Location N1	N2	N3 or more distant
5 (4)[a]	2 (1)	3 (3)	0 (0)
6 (5)	3 (2)	3 (3)	0 (0)
22 (5)	7 (3)	6 (0)	9 (2)
44 (7)	10 (4)	8 (0)	26 (3)
14 (3)	9 (2)	3 (1)	2 (0)
11 (11)	5 (5)	3 (3)	3 (3)
9 (9)	4 (4)	5 (5)	0 (0)
11 (9)	4 (4)	5 (3)	2 (2)
6 (6)	4 (4)	1 (1)	1 (1)
21 (9)	9 (5)	6 (4)	6 (0)
22 (9)	3 (3)	5 (4)	14 (2)
17 (14)	4 (3)	10 (8)	3 (3)
14 (11)	3 (2)	7 (5)	4 (4)
202 (102)	67 (42)	65 (40)	70 (20)

[a]Numbers in parentheses indicate positive lymph nodes.

CONCLUSION

RT-PCR for amplification of CEA and CK-20 mRNAs for detecting micrometastases from colorectal carcinomas in lymph nodes is a powerful and sensitive method. At present, the relationship between the results and prognosis is still controversial, but the increase in accuracy of clinical staging and indications for surgery and chemotherapy which are potentially achievable suggests that wider adoption of this approach is warranted. Accumulation of data should allow improvements in choice of appropriate therapeutic modes in the future.

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